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Negative-ion MALDI-MS^2 for discrimination of α2,3- and α2,6-sialylation on glycopeptides labeled with a pyrene derivative☆

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ABSTRACT

Here, we propose a novel method for the discrimination of α2,3- and α2,6-sialylation on glycopeptides. To stabilize the sialic acids, the carboxyl moiety on the sialic acid as well as the C-terminus and side chain of the peptide backbone were derivatized using 1-pyrenyldiazomethane (PDAM). The derivatization can be performed on the target plate for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), thereby avoiding complicated and time-consuming purification steps. After the on-plate PDAM derivatization, samples were subjected to negative-ion MALDI-MS using 3AQ-CHCA as a matrix. Deprotonated ions of the PDAM-derivatized form were detected as the predominant species without loss of sialic acid. The negative-ion collision-induced dissociation (CID) of PDAM-derivatized isomeric sialylglycopeptides, derived from hen egg yolk, showed characteristic spectral patterns. These data made it possible to discriminate α2,3- and α2,6-sialylation. In addition, sialyl isomers of a glycan with an asparagine could be discriminated based on their CID spectra. In brief, the negative-ion CID of PDAM-derivatized glycopeptides with α2,6-sialylation gave an abundant ^{0,2}A-type product ion, while that with α2,3-sialylation furnished a series of ^{2,4}A/Y-type product ions with loss of sialic acids. The unique fragmentation behavior appears to be derived from the difference of pyrene binding positions after ionization, depending on the type of sialylation. Thus, we show that on-plate PDAM derivatization followed by negative-ion MALDI-MS^2 is a simple and robust method for the discrimination of α2,3- and α2,6-sialylation on glycopeptides.

1. Introduction

Both electrospray ionization mass spectrometry (ESI-MS) [1] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [2–5] have proven to be powerful analytical tools for characterizing biomolecules. MALDI-MS has several advantages over other mass spectroscopic techniques due to the relative simplicity of spectral interpretation, rapid analysis and repeated measurement of the same sample. Thus, MALDI-MS has been used in a wide variety of applications for the analysis of glycans [6–8]. In particular, a collision-induced dissociation (CID) technique combined with MALDI-MS will be indispensable for the structural determination of glycans because of the plethora of isomeric structures.

Sialic acids exist on many glycoconjugates, such as glycoproteins and glycolipids. Given that sialylated glycans of glycoconjugates play an important role in various biological functions, determination of their precise structures, including the type of sialyl linkages, is essential. However, determination of the type of sialyl linkage by MS is still a challenging task because of preferential detachment of sialic acid residues from the glycan. This detachment usually occurs not only in the CID conditions but also in the ionization events in MALDI. As a result, structural information of sialylated glycan is lost. Various approaches have been reported to prevent loss of sialic acid, such as the use of a cool matrix [9–13] and measurement by high-pressure MALDI [14,15]. Although these approaches are effective for MS^1 experiments, the sialic acids are preferentially detached under CID, which is needed for structural determination. It is believed that a dissociative proton of the carboxyl moiety on sialic acid leads to the loss of the sialic acid unit. To eliminate the dissociative carboxylic proton, sialic acid could be converted to its ester [16,17], amide [18] or derivatized with acetohydrazide [19]. Indeed, these methods enabled us to detect sialylated glycans in positive-ion mode without a loss of sialic acid. Unfortunately, however, the type of sialyl linkage could not be identified. Permethylation [20–22] and perbenzylation [23] has been reported to be effective for discrimination of α2,3- and α2,6-sialylation by preferential formation of lactone from α2,3-linked sialic acid. Harvey et al. introduced a unique methyl ester derivatization for discrimination of α2,3- and α2,6-sialylation on the glycan from the MALDI

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mass spectrum without MS^n experiments [24]. Although these approaches may be applicable to released glycans, there are currently no reports concerning the stabilization of sialic acid and discrimination of the type of sialyl linkage on glycopeptides.

Since the original results reported by Biemann and Martin [25], positively charged ions and their product ions have been used for amino-acid sequencing of peptides and proteins. However, scant attention has been paid to negatively charged ions. The structural determination of glycans based on MS^n has also been performed in positive-ion mode because glycans usually ionize as $[M+Na]^+$ or $[M+K]^+$. Recently, CID of negatively charged ions has proved to be effective for the structural determination of glycans [26–33]. In particular, we have focused on the analysis of both neutral and acidic glycans in negative-ion mode, rather than positive-ion mode, because negative-ion CID of glycans derivatized with pyrene generate unique product ions for structural determination [29,30]. In general, negative-ion formation from neutral glycan is difficult, showing no or low ion yields. By contrast, we found that glycans derivatized with pyrene produced an abundance of ions compared with other derivatization protocols such as 2-aminopyridine [29]. In order to obtain higher yields of negative ions, on-plate pyrene derivatization for glycans has been developed as a simple and rapid method [34]. Furthermore, we have developed another unique on-plate pyrene derivatization for glycopeptides [35]. The method not only enhances ion yield of glycopeptides, but also suppresses a loss of sialic acids on glycans and glycopeptides. Previously, by using this pyrene derivatization method, we could discriminate α2,3- and α2,6-sialylation on released glycans by MALDI-MS^2 [36]. In the present study, we succeeded in discriminating glycopeptides with sialylated isomers based on their negative-ion MS^2 spectra. In contrast to the analysis of released glycans, glycopeptide analysis provides site-specific information of glycosylation in addition to the possible identification of the corresponding protein. The determination of altered patterns of sialylation in glycopeptides is indispensable for the elucidation of their biological function. This is the first report to describe a highly sensitive and straightforward method of discriminating α2,3- and α2,6-sialylation on glycopeptides by MALDI-MS.

2. Experimental

2.1. Materials

A sialylglycopeptide (SGP) was prepared from hen egg yolk [37]. α-2,3-Sialyltransferase (EC: 2.4.99.4) from *Photobacterium phosphoreum* JT-ISH-224 strain and α-2,6-sialyltransferase (EC: 2.4.99.1) from *Photobacterium damselae* JT160 strain were purchased from Japan Tobacco Inc. (Tokyo, Japan). CMP-NeuAc was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Cellulose fibrous medium was purchased from Sigma–Aldrich (Steinheim, Germany). NuTip Carbon was purchased from Glygen Corp. (Columbia, MD). Four kinds of sialylated isomers of disialylated glycans attached to an asparagine (A2-Asn) were purchased from Otsuka Chemical (Tokushima, Japan). The highly purified MALDI matrix chemicals, 2,5-dihydroxybenzoic acid (DHBA) and α-cyano-4-hydroxycinnamic acid (CHCA), were purchased from Shimadzu-Biotech (Kyoto, Japan); and 3-aminoquinoline (3AQ) was purchased from Sigma–Aldrich; 1,5-diaminonaphtalene (1,5-DAN) was purchased from WAKO Pure Chemical, Inc. (Osaka, Japan). 1-Pyrenyldiazomethane (PDAM) was purchased from Molecular Probes, Inc. (Eugene, OR). The solvents, ethanol (EtOH, LC/MS grade), acetonitrile (MeCN, LC/MS grade) and butanol (HPLC grade), were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan); toluene (HPLC grade) and xylene (HPLC grade) were purchased from Sigma–Aldrich. The water used in all experiments was purified by using a NANOpure DIAMOND Ultrapure Water System from Barnstead (Boston, MA). All reagents were used without further purification.

2.2. Preparation of glycopeptides with α2,3- or α2,6-linked sialic acid

For desialylation, a purified SGP derived from hen egg yolk was heated in 0.8% TFA at 80 °C for 45 min. Then, sialic acids were added on the non-reducing ends of desialylated SGP glycans with specific enzymes. The enzyme reaction was performed according to the protocol described by Yamamoto et al. [38,39] with slight modification. The 150 μL reaction mixture consisted of 20 mU of each enzyme solution (α-2,3-sialyltransferase or α-2,6-sialyltransferase), 500 pmol of asialo-SGP, 2.3 mM CMP-NeuAc, 20 mM bis–Tris buffer (pH 6.0), 0.5 M NaCl and 0.03% Triton X-100. The reaction was carried out at 37 °C for 4 h. Under this condition, both disialylated and monosialylated SGP were obtained and confirmed by using different type of MALDI instrument in linear mode, which can prevent loss of sialic acid. The structures of desialylated SGP and synthesized SGP are shown in Fig. 1.

The glycopeptides were isolated by using the hydrophilic affinity method [40]. Reaction products were dissolved in an organic solvent mixture containing butanol/ethanol/water (4:1:1 by volume). 30 mg of cellulose fibrous medium was washed in water, ethanol/water (1:1 by volume), and conditioned with butanol/ethanol/water (4:1:1 by volume). After conditioning, cellulose was suspended with the 400 μL of organic solvent mixture. A 20 μL aliquot of the suspension was then added to the reaction product solution. The resulting mixtures were shaking with a vortex mixer for 1 h and then centrifuged for 2 min. The supernatants were removed and the cellulose was washed five times with the 300 μL of the same organic solvent. The samples were extracted with a vortex mixer the cellulose in 200 μL of ethanol/water (1:1, by volume) for 15 min. The samples were then centrifuged, and the supernatants transferred to another microtube. The same extraction procedure was repeated four times. Finally, the supernatants were completely dried using a Speed Vac and reconstituted with 20 μL of 0.1% TFA.

Cellulose-purified reaction products were further desalted by NuTip carbon. Before loading the sample, the NuTip Carbon was conditioned by aspirating 20 μL of the releasing solution, 80% MeCN with 0.1% TFA, followed by the binding solution, 0.1% TFA. Cellulose-purified reaction products were loaded on the NuTip Carbon by aspirating 20 times. Then the NuTip Carbon was washed with 20 mL of binding solution by aspirating 20 times, and the samples were eluted by expelling the releasing solution 10 times. The same releasing procedure was repeated 10 times. The released solution were completely dried on a Speed Vac and reconstituted with water.

2.3. Pyrene derivatization on the target plate and mass spectrometry

Analyte solution was first deposited on a mirror-polished stainless-steel MALDI target and left to dry. A fresh solution of 0.25 μL of PDAM 10 nmol/μL in dimethyl sulfoxide was added onto the dried analyte and the plate was heated at 80 °C for 5 min. The plate was then rinsed with toluene or xylene to remove excess PDAM and briefly dried under vacuum. Subsequently, the matrix solution, DHBA or 3AQ-CHCA was applied. DHBA was dissolved in 60% MeCN/H_2O to a concentration of 10 mg/mL. Then the 1 μL of resulting solution was applied onto the plate and left to dry. A stock solution of 3AQ-CHCA liquid matrix was prepared by dissolving 35 mg 3AQ in 150 μL of a saturated solution of CHCA in MeOH [41]. A stock solution of 3AQ-CHCA was diluted by a factor of 10 in the 60% MeCN/H_2O and 1 μL of resulting solution was applied onto the plate. MALDI-TOF mass spectra and MS^n spec-

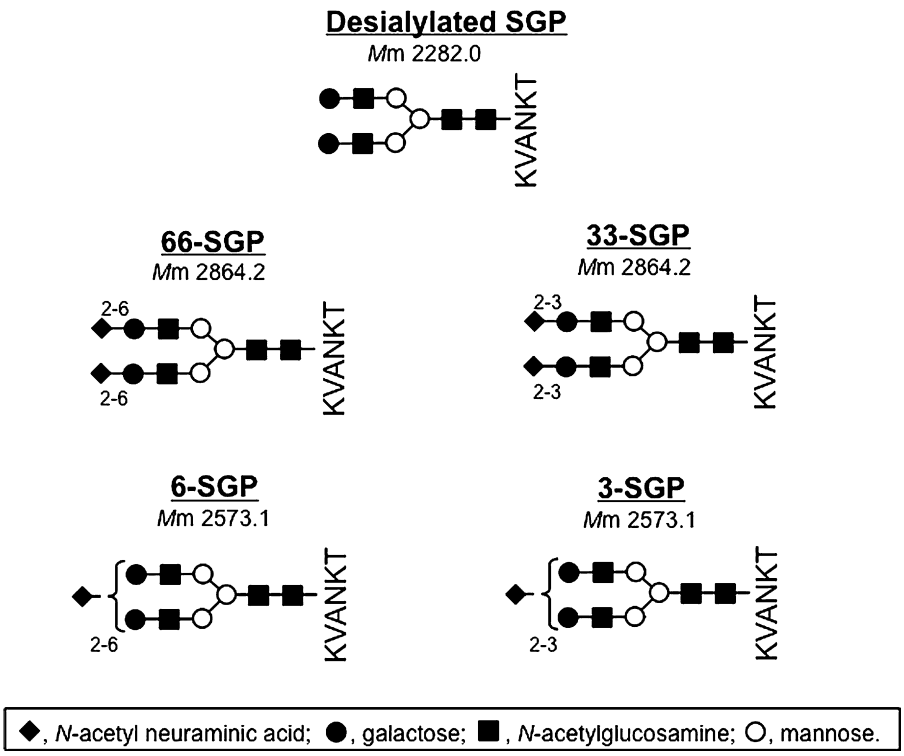

Fig. 1. Structures of desialylated SGP and synthesized SGP from hen egg yolk.

tra were acquired using an AXIMA-QIT instrument consisting of a quadrupole ion trap and reflector time-of-flight analyzer (Shimadzu Biotech, Manchester, U.K.). A nitrogen laser (337 nm) was used to irradiate the sample for ionization. Spectra were obtained in positive- and negative-ion mode.

3. Results and discussion

3.1. The effect of on-plate PDAM derivatization

Due to the presence of a dissociative proton of the carboxyl group on the sialic acid, sialylated glycans often show cleavage of sialyl linkages and loss of sialic acids during both CID and the ionization event of MALDI. Additionally, considerable metastable fragmentation was observed when the AXIMA-QIT instrument was used in MS^1 mode. For instance, the negative-ion MS of disialylated glycan mainly showed a monosialylated ion $[M\text{-}Neu5Ac\text{-}H]^-$, indicating an extensive loss of sialic acid (data not shown). As can be seen in Fig. 2, disialylated glycopeptide (SGP) gave the desialylated ion $[M\text{-}2Neu5Ac\text{-}H]^-$ at m/z 2281.0 and the monosialylated ion $[M\text{-}Neu5Ac\text{-}H]^-$ at m/z 2572.1 with poor signal intensity but no disialylated ion. These observations show that most of the sialic acid is detached before detection. The aim of the present study was to develop a method to discriminate between different sialyl linkages by MALDI-MS^2. The loss of sialic acid in MS^1 mode should therefore be avoided.

Here, we used PDAM derivatization to stabilize the sialyl linkages of the sialylated glycopeptides (Fig. 3). PDAM readily reacts with carboxylic acids even in the absence of a catalyst [42,43]. As a result of ester formation, the mass of analyte increases by 214.1 Da for every free carboxylic acid that is subject to derivatization. In this paper, we represent the PDAM-derivatized forms with 1, 2 and 3 pyrene molecules as M*, M** and M***, respectively. PDAM derivatization is expected to have two advantages in terms of our glycopeptide analysis; (i) prevents loss of sialic acid by removal of dissociative protons, (ii) enhances ionization on MALDI due to the highly hydrophobic and aromatic nature of the pyrene moiety [29]. This derivatization can be rapidly performed on the target plate, thereby avoiding time-consuming purification steps prior to MALDI-MS, along with the inevitable loss of analyte.

Derivatized sialylated glycans were expected to be easily ionized as $[M+H]^+$ or $[M+Na]^+$, as is the case for neutral glycans [16–24]. Therefore, SGP derivatized with PDAM was initially subjected to measurement in positive-ion mode. SGP possesses three carboxyl groups on the sialic acids and the C-terminus of the peptide backbone that can be readily derivatized. However, no peak was observed at the expected m/z values corresponding to PDAM-derivatized SGP, such as $[M^{***}+H]^+$, $[M^{***}+Na]^+$ or $[M^{***}+K]^+$. Instead, PDAM-derivatized SGP gave very intense peaks corresponding to the desialylated ion $[M\text{-}2Neu5Ac\text{-}H]^-$ at m/z 2281.0 and the monosialylated ion $[M\text{-}Neu5Ac\text{-}H]^-$ at m/z 2572.1 in negative-ion MALDI (Fig. 2b). Moreover, the loss of sialic acids could not be suppressed. Other weak signals corresponding to $[M^*\text{-}Neu5Ac\text{-}H]^-$ (m/z 2786.2) and fragment ions were also detected. As indicated in the comparison between Fig. 2a and b, the negative-ion MS of underivatized and PDAM-derivatized SGP were very similar. Interestingly, however, the ion yields were clearly enhanced after on-plate PDAM derivatization. Thus, the ion yields of $[M\text{-}2Neu5Ac\text{-}H]^-$ and $[M\text{-}Neu5Ac\text{-}H]^-$, which lack a pyrene moiety, were greatly enhanced by on-plate PDAM derivatization. We reasoned that if derivatization had not taken place the ion yields would have been unaffected.

We considered the possibility that the enhanced ion yields might be attributed to a co-matrix effect of PDAM, but eliminated the possibility. The ion yield of neutral glycan, which does not react with PDAM due to the lack of a carboxyl moiety, was not enhanced by the procedure of on-plate PDAM derivatization (data not shown). Thus, the derivatization reaction is likely to be essential for enhancement of ionization. We therefore assumed that although the three carboxylic acids of SGP had been completely derivatized by PDAM, pyrene moieties were subsequently released by in-source decay (ISD) during the ionization event. This assump-

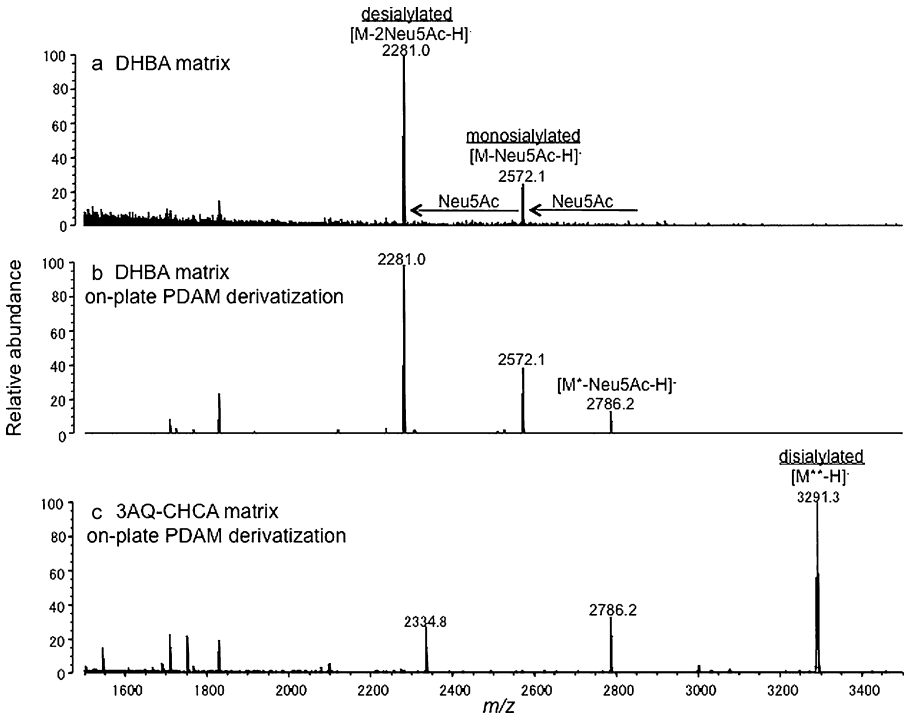

Fig. 2. Negative-ion mass spectra of (a) underivatized and (b and c) PDAM-derivatized SGP obtained with (a and b) DHBA and (c) 3AQ-CHCA as matrices. M* refers to a PDAM-derivatized form. The number of asterisks indicates the number of pyrene groups introduced.

tion is supported by the fact that the highly PDAM-derivatized forms can be readily detected by using an alternative type of matrix. Indeed, using 3AQ-CHCA as a matrix the release of the pyrene moiety by ISD is suppressed. Consequently, the PDAM-derivatized ion could be observed without loss of sialic acids (Fig. 2c). Nonetheless, release of the pyrene moiety by ISD could still be detected. Therefore, the intense peak corresponding to $[M^{**}\text{-}H]^-$ at m/z 3291.3 was observed in negative-ion mode due to the presence of a negative charge on a free carboxylic acid. Hence, the use of 3AQ-CHCA can inhibit, but not completely suppress, the ISD-induced release of pyrene. By contrast, the release of the pyrene moiety was greatly enhanced by using 1,5-DAN as a matrix. 1,5-DAN matrix is reported to be better suited to MALDI-ISD experiments than DHBA [44]. By using 1,5-DAN as a matrix, pyrene moieties were completely released resulting in a dominant peak at m/z 2281.0 (data not shown), corresponding to desialylated ion $[M\text{-}2Neu5Ac\text{-}H]^-$. This observation supports the idea that the release of a pyrene moiety is due to the ISD reaction, rather than a post-source decay (PSD) event. Indeed, using an appropriate matrix that can completely prevent ISD reactions, it might be possible to detect the triply derivatized form in positive-ion MALDI. Unfortunately, to date, no such matrix has been found. Nonetheless, our studies showed that the ion of $[M^{**}\text{-}H]^-$ is useful as a precursor of MS^2 experiments for discrimination of α2,3- and α2,6-sialylation, as described in the next section.

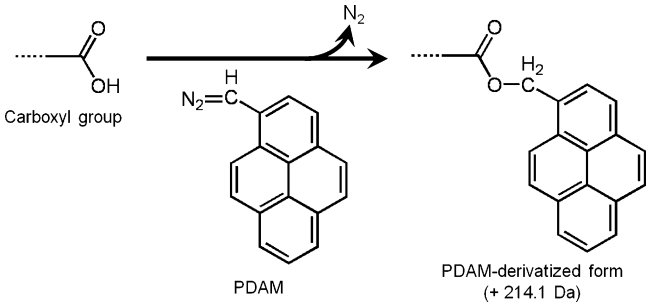

Fig. 3. Reaction of PDAM derivatization.

It should be noted that although a combination of on-plate PDAM derivatization and the use of 3AQ-CHCA as a matrix certainly stabilizes the sialyl linkages, some loss of sialic acid is still observed. The ion at m/z 2786.2 in the Fig. 2c is generated by the loss of sialic acid and pyrene moiety from the $[M^{**}\text{-}H]^-$. Therefore, the method would be somewhat limited in profiling mixtures containing glycans with different number of sialic acids. Alternatively, the measurement by other type of MALDI instrument in linear mode, which can prevent loss of sialic acid, would provide more accurate profiling results.

3.2. Discrimination of sialyl linkages on SGP based on MS^n spectra

The sialylated glycopeptides, 66-SGP, 6-SGP, 33-SGP and 3-SGP in Fig. 1 were synthesized from desialylated SGP by enzymatic reaction. The structural difference between 66-SGP and 33-SGP is confined to the type of sialic acid linkage on both the α1-6 and α1-3 antennae. 6-SGP and 3-SGP possess a sialic acid on the glycan moiety, but it is unclear which antenna is sialylated.

After on-plate PDAM derivatization, by using 3AQ-CHCA as a matrix, the intact ions were observed without significant loss of sialic acids. Disialylated SGP, 66-SGP and 33-SGP, were detected as $[M^{**}\text{-}H]^-$ (m/z 3291.3) reminiscent of Fig. 2c. The ions were then selected as a precursor for CID experiments. Clearly, each spectral pattern is not the same, enabling easy discrimination of α2,3- and α2,6-sialylation (Fig. 4). The CID of 66-SGP produced an intense peak corresponding to the $^{0,2}A_7$-type product ion with a pyrene moiety at m/z 2334.9 (Fig. 4a) (fragmentation nomenclature by Domon and Costello [45]). In addition, the loss of sialic acid was also observed at very low abundance (m/z 3000.4). In contrast, the series of product ions corresponding to $^{2,4}A/Y$-type cleavage were predominantly observed for 33-SGP (Fig. 4b). In the same way, monosialylated SGP were derivatized and analyzed in negative-ion mode. Monosialylated SGP, 6-SGP and 3-SGP were ionized as $[M^*\text{-}H]^-$ (m/z 2786.2) and then subsequently selected as precur-

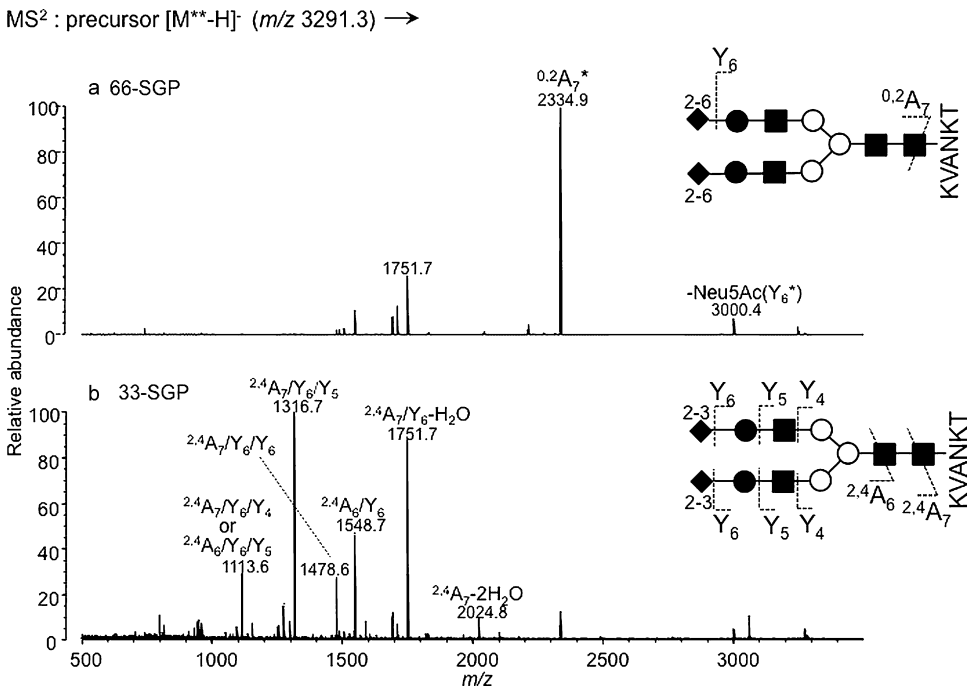

Fig. 4. Negative-ion CID spectra of PDAM-derivatized (a) 66-SGP and (b) 33-SGP. The peaks of $[M^{**}\text{-}H]^-$ (*m*/*z* 3291.3) were selected as a precursor for CID experiments. Asterisk indicates a PDAM-derivatized form. The main product ions are illustrated in the inset without distinguishing between the upper (α1-6) or lower (α1-3) branch.

sors for CID experiments. As in the case of disialylated SGP, the CID spectra of 6-SGP and 3-SGP were quite different from each other, enabling easy discrimination of α2,3- and α2,6-sialylation (Fig. 5). The CID of 6-SGP produced an intense peak, corresponding to the ^{0,2}A-type product ion at *m*/*z* 1829.8 (Fig. 5a), while that of 3-SGP predominantly gave a series of ions corresponding to ^{2,4}A/Y-type product ions at *m*/*z* 1478.6, 1275.6 and 1113.6 (Fig. 5b). In brief, CID of SGP with α2,6-sialylation (66- and 6-SGP) gave an intense peak corresponding to the ^{0,2}A-type product ion, while that with α2,3-sialylation (33- and 3-SGP) produced a series of ^{2,4}A/Y-type product ions with loss of sialic acids. For comparison, the negative-ion CID spectra of underivatized 6-SGP and 3-SGP are shown in Fig. 6. Without PDAM derivatization, the preferential loss of sialic acid was observed on both isomers and it was impossible to discriminate sialyl isomers from the spectral data. Unfortunately, the negative-ion CID spectra of underivatized 66-GSP and 33-SGP could not be obtained because of an extensive loss of sialic acids in MS^1 mode (see Fig. 2a). Thus, PDAM derivatization followed by a negative-ion CID experiment was found to stabilize the sialyl linkages and help discriminate sialyl isomers.

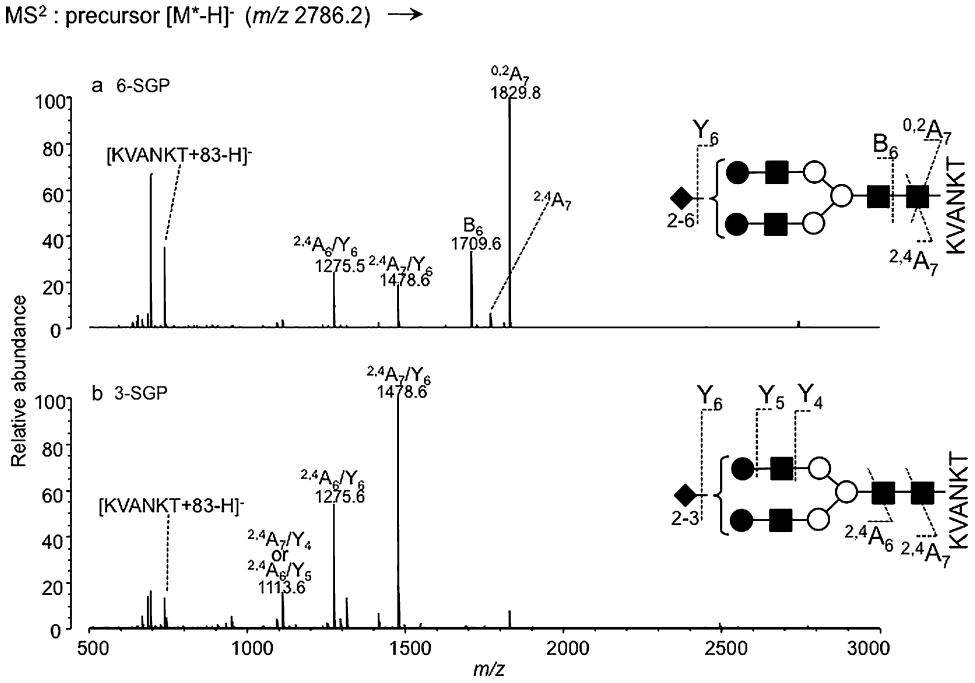

Fig. 5. Negative-ion CID spectra of PDAM-derivatized (a) 6-SGP and (b) 3-SGP. The peaks of $[M^*\text{-}H]^-$ (*m*/*z* 2786.2) were selected as a precursor for CID experiments. Asterisk indicates a PDAM-derivatized form. The main product ions are illustrated in the inset without distinguishing between the upper (α1-6) or lower (α1-3) branch.

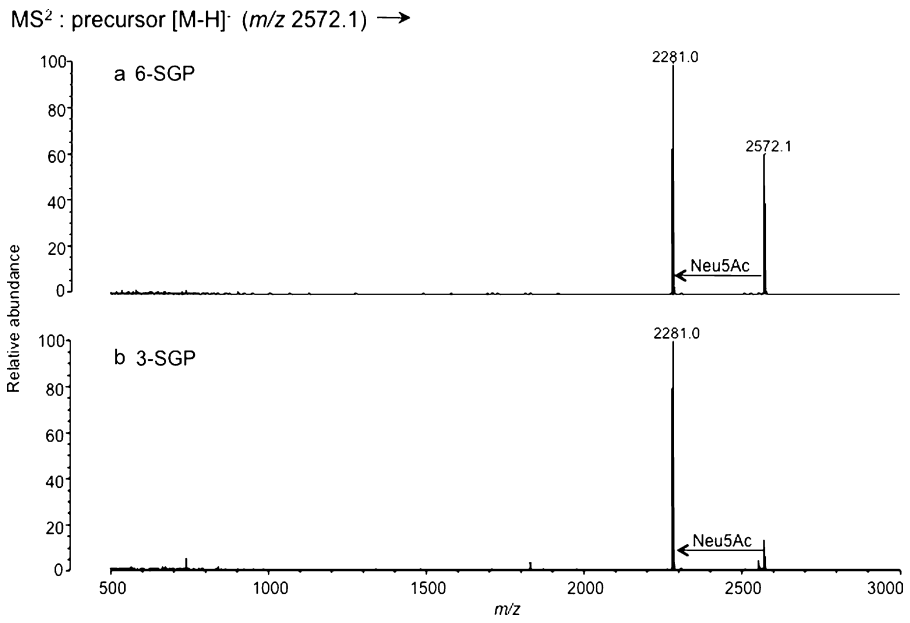

Fig. 6. Negative-ion CID spectra of deprotonated (a) 6-SGP and (b) 3-SGP. Deprotonated ion $[M-H]^-$ was selected as a precursor.

To understand and rationalize the difference of the fragmentation behavior, it is necessary to consider the derivatized structures of the precursor ions after on-plate PDAM derivatization. It should be noted that although 66-SGP and 33-SGP have a total of three carboxyl groups (i.e., two sialic acids plus the C-terminus of the peptide backbone), only the PDAM-derivatized form with two pyrene moieties were detected. One pyrene moiety is presumably released during ionization, probably by ISD. Therefore, from the precursor ions, $[M^{**}-H]^-$, two different structures (form A or B in Fig. 7), are possible depending on the location of the pyrene group. In the CID spectrum of 66-SGP, the ^{0,2}A-type product ion with a pyrene moiety at m/z 2334.9, which is generated by a neutral loss of 956.4 Da from precursor $[M^{**}-H]^-$ (m/z 3291.3), is mainly observed. The production of the ion provides direct evidence for the existence of a pyrene group on the sialic acid of the glycan. In addition, the neutral loss of 956.4 Da (KVANKT + 83 + 214.1 Da) indicates the C-terminus of the peptide backbone is derivatized by PDAM. Hence, it is very likely that the derivatized 66-SGP ionizes as form A (i.e., one pyrene group is introduced to the peptide and one to the glycan moiety). However, the ^{0,2}A-type product ion was not a major product ion for 33-SGP. In general, it has been reported that negative-ion CID of sialylated or sulfated glycopeptides provides abundant ^{0,2}A-type product ions [46,47]. This can be attributed to negative charge localization on the sialic acid or sulfate group. Nevertheless, CID of 33-SGP predominantly generated a series of product ions corresponding to ^{2,4}A/Y-type product ions, rather than a ^{0,2}A-type product ion. By negative-ion CID, ^{2,4}A-type product ions were obtained from desialylated SGP (data not shown). Negative-ion CID of glycopeptides with neutral glycan often generates ^{2,4}A-type product ions [47]. Hence, the production of ^{2,4}A/Y-type product

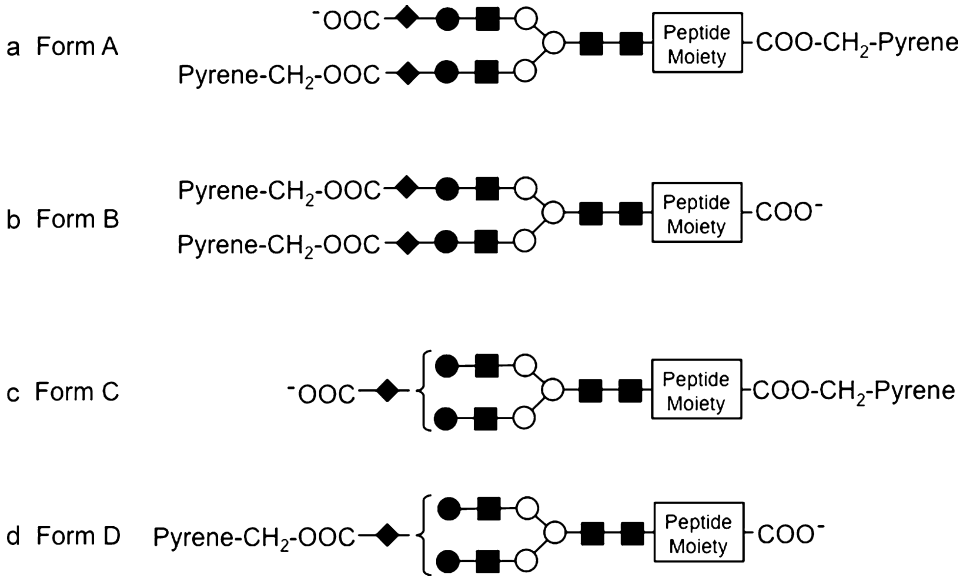

Fig. 7. Possible ion structures of PDAM-derivatized glycopeptides detected in negative-ion MALDI-MS. (a and b) Disialylated glycopeptide, (c and d) monosialylated glycopeptide. PDAM-derivatized position and negatively charged position are shown without distinguishing between the upper (α1-6) or lower (α1-3) branch.

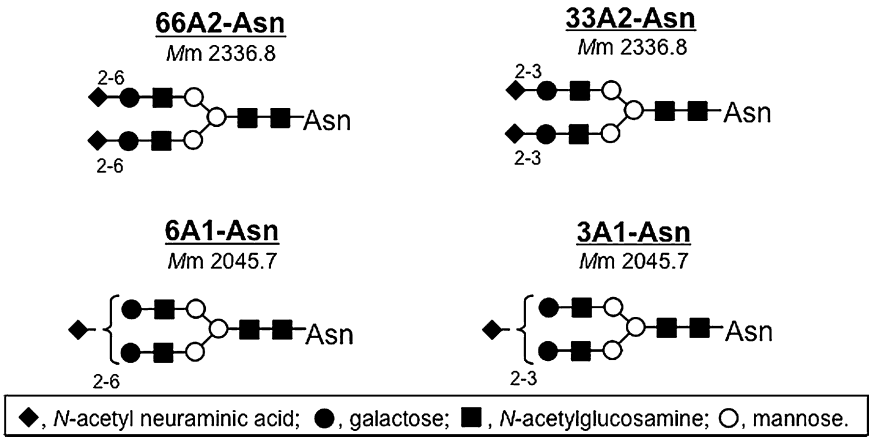

Fig. 8. Structures of A2-Asn and A1-Asn.

ions, rather than the ^{0,2}A-type product ion, indicates an absence of strong negative charge on the glycan moiety. These observations suggest both sialic acids on 33-SGP are derivatized by PDAM, resulting in a neutral glycan. Therefore, 33-SGP is probably ionized as form B (i.e., two pyrene groups bound to both sialic acids of the glycan moiety).

In the case of monosialylated SGP, both 6-SGP and 3-SGP were detected as $[M^*\text{-}H]^-$, indicating that either a sialic acid or the C-terminus was derivatized. Therefore, the precursor ions $[M^*\text{-}H]^-$ show two possible structures, form C and D, according to its pyrene-binding position (Fig. 7c and d). In the CID spectrum of 6-SGP, the ^{0,2}A-type product ion at *m/z* 1829.8, generated by a neutral loss of 956.4 Da from the precursor ion $[M^*\text{-}H]^-$ at *m/z* 2786.2, was observed as the base peak. The generation of this product ion suggests the presence of a pyrene moiety on the C-terminus of the peptide backbone. It is therefore clear that PDAM-derivatized 6-SGP mainly ionizes as form C. By contrast, the intensity of the peak corresponding to the ^{0,2}A-type product ion was greatly reduced in the CID spectrum of 3-SGP derivatized with PDAM. Instead, the CID of 3-SGP gave a series of product ions, principally corresponding to ^{2,4}A/Y-type ions. The fragmentation behavior of the ^{0,2}A-type or ^{2,4}A/Y-type product ions was consistent with that of 66-SGP or 33-SGP. Therefore, it is possible that 3-SGP is ionized as form D. Additional experiments are needed to clarify the pyrene binding positions and rationalize the fragmentation behavior.

3.3. Discrimination of sialyl linkages on A2- and A1-Asn based on MS^2 spectra

In order to verify the position of pyrene binding and confirm that this unique fragmentation behavior is not specific for the peptide sequence (KVANKT), simple glycopeptides comprising glycans with an asparagine (A2-Asn and A1-Asn) were derivatized and analyzed in negative-ion mode. The structures of A2-Asn and A1-Asn are illustrated in Fig. 8. 66A2-Asn or 33A2-Asn possess α2,6- or α2,3-sialylations on both α1-3 and α1-6 antennae, respectively.

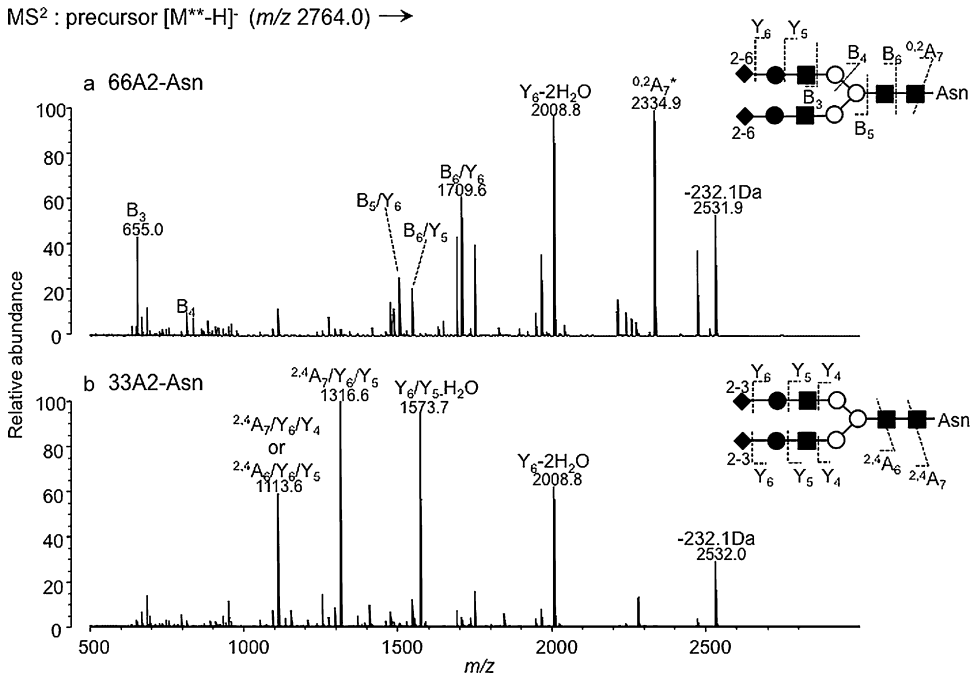

Fig. 9. Negative-ion CID spectra of PDAM-derivatized (a) 66A2-Asn and (b) 33-Asn. The peaks of $[M^{**}\text{-}H]^-$ (*m/z* 2764.0) were selected as a precursor for CID experiments. Asterisk indicates a PDAM-derivatized form. The main product ions are illustrated in the inset without distinguishing between the upper (α1-6) or lower (α1-3) branch.

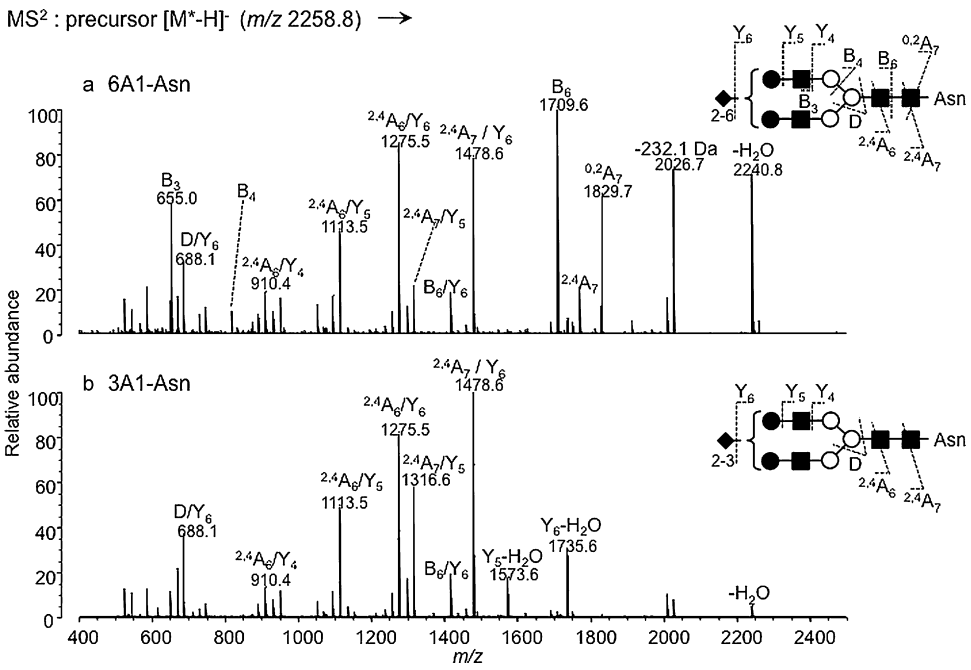

Fig. 10. Negative-ion CID spectra of PDAM-derivatized (a) 6A1-Asn and (b) 3A1-Asn. The peaks of $[M^*\text{-}H]^-$ (*m*/*z* 2258.8) were selected as a precursor for CID experiments. Asterisk indicates a PDAM-derivatized form. The main product ions are illustrated in the inset without distinguishing between the upper (α1-6) or lower (α1-3) branch.

The monosialylated forms, 6A1-Asn or 3A1-Asn possess α2,6- or α2,3-sialylation on either the α1-3 or α1-6 antenna. However, it was unclear which antenna was sialylated.

After PDAM derivatization using the 3AQ-CHCA matrix, A2-Asn and A1-Asn were ionized as $[M^{**}\text{-}H]^-$ at *m*/*z* 2764.0 and $[M^*\text{-}H]^-$ at *m*/*z* 2258.8, respectively. The CID spectra of isomeric A2-Asn were derived from the ion of $[M^{**}\text{-}H]^-$ as a precursor (*m*/*z* 2764.0) under the same CID conditions (Fig. 9). The negative ion CID of PDAM-derivatized isomeric A2-Asn showed quite different spectral patterns depending on the nature of the sialyl linkages, even if the glycan is attached exclusively to Asn. The ion at *m*/*z* 2531.9, corresponding to the neutral loss of pyrenyl methyl alcohol (232.1 Da), was commonly observed in both spectra. Also, the ion at *m*/*z* 2008.8 was commonly observed, which could be assigned to the product ion of Y_6-2H_2O. In addition to these peaks, the pattern of product ion formation is entirely consistent with that of disialylated SGP, 66-SGP and 33-SGP. The product ion at *m*/*z* 2334.9 by neutral loss of 429.1 Da (Asn + 83 + 214 Da), which was exclusively observed in the CID of 66A2-Asn, was assigned to the $^{0,2}A_7$-type product ion with one pyrene moiety (Fig. 9a). The production of $^{0,2}A_7$-type product ion with one pyrene group indicates that 66A2-Asn is ionized reminiscent of form A in Fig. 7. The ^{2,4}A/Y-type product ions were mainly observed in the CID of 33A2-Asn (Fig. 9b). As discussed earlier, the generation of ^{2,4}A/Y-type product ions is favored by CID of glycopeptides with a neutral glycan. Negative ion formation of derivatized 33A2-Asn appeared to occur by a process reminiscent of form B in Fig. 7.

The CID spectra of the monosialylated form showed a slightly different spectral pattern (see Fig. 10). The negative-ion CID of derivatized 6A1-Asn provided both $^{0,2}A_7$ and ^{2,4}A/Y-type product ions. The production of B_3, B_4, B_6, $^{2,4}A_7$ and $^{0,2}A_7$ indicates that the structure of the negative ion of derivatized 6A1-Asn is form C. However, the CID of 3A1-Asn was similar but not identical to that obtained from 6A1-Asn. The critical difference between the two spectra is that $^{0,2}A_7$, $^{2,4}A_7$ and B_6-type product ions are never observed in the CID of 3A1-Asn. The detection of these ions could be attributed to the presence of a negative charge on the sialic acid unit. Therefore, the absence of these ions from the CID spectrum of 3A1-Asn suggests that a negative charge is not present on the sialic acid moiety because the carboxylic acid is esterified and neutralized by PDAM derivatization. In fact, without PDAM derivatization, $^{0,2}A_7$-type product ions are commonly observed in the negative-ion CID spectra of underivatized 6A1-Asn and 3A1-Asn (Fig. 11). It is therefore likely that the negative ion structure of derivatized 3A1-Asn is form D.

It has been reported that α2,3-sialyl linkage cleaved much more readily than α2,6-sialyl linkage on the sialyllactose isomers using the negative-ion MALDI-PSD fragmentation method [48]. However, in our results, the CID spectrum of underivatized 3A1-Asn was very similar to that of 6A1-Asn (compare Fig. 11a and b). This indicates that the stability difference of α2,3-linked and α2,6-linked sialic acid residues on A1-Asn is not reflected in their respective CID spectral patterns. Hence, the difference in stability is insufficient to discriminate between the types of sialyl linkages in the analyzed samples. The successful discrimination of different types of sialyl linkages on glycopeptides using the PDAM derivatization procedure must be caused by other factors. Our results suggest that the heterogeneity of the pyrene binding position on the glycopeptide ions after ISD is the key factor that enables us to discriminate α2,3- and α2,6-sialylation on glycopeptides based on their CID spectra.

In brief, the negative-ion CID of PDAM-derivatized glycopeptides possessing only α2,6-sialylation (66-SGP, 6-SGP, 66A2-Asn and 6A1-Asn) gave a $^{0,2}A_7$-type product ion in abundance, while those glycopeptides possessing only α2,3-sialylation (33-SGP, 3-SGP, 33A2-Asn and 3A1-Asn) showed intense $^{2,4}A_7$/Y-type product ions with the loss of sialic acid and pyrene moiety. The unique fragmentation behavior seems to be derived from the difference of pyrene-binding positions after ISD during the ionization event on MALDI. We assume that the efficiency of the derivatization reaction is almost the same for carboxyl groups on α2,3-linked sialic acid, α2,6-linked sialic acid and at the C-terminus of the peptide backbone because negative-ion yields of PDAM-derivatized 33A2-Asn and 66A2-Asn were almost identical. The selectivity of the pyrene-binding position, which results in the unique fragmenta-

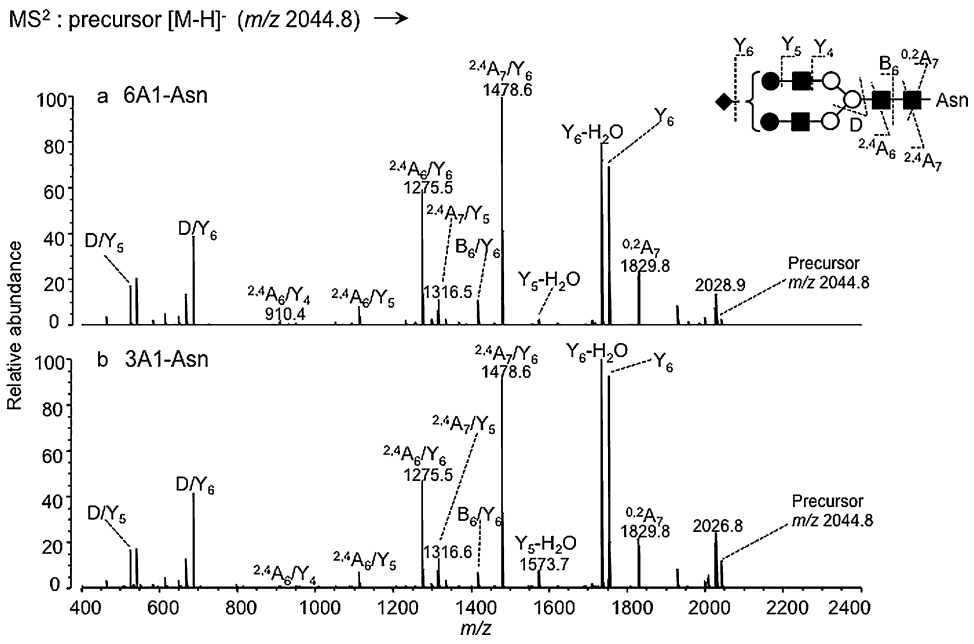

Fig. 11. Negative-ion CID spectra of underivatized (a) 6A1-Asn and (b) 3A1-Asn. The peaks of $[M-H]^-$ (m/z 2044.8) were selected as a precursor for CID experiments. The main product ions are illustrated in the inset without distinguishing between the upper (α1-6) or lower (α1-3) branch.

tion pattern, appears to be due to differences in the extent of pyrene release from the derivatized forms by ISD. The order of pyrene release is thought to be as follows: pyrene on the α2,6-linked sialic acid > on the C-terminus > on the α2,3-linked sialic acid. We are currently investigating the detailed mechanism of pyrene release by ISD. Regardless of the precise mechanism of pyrene release, the PDAM derivatization method followed by negative-ion MALDI-MS^2 is a highly effective tool for the discrimination of different types of sialyl linkages on glycopeptides.

4. Conclusions

In the present work, we have developed a new approach for discriminating between α2,3- and α2,6-sialylation of glycopeptides. On-plate PDAM derivatization followed by negative-ion MALDI-MS^2 was used to analyze a series of glycopeptide samples. The derivatization procedure is a simple and rapid method that prevents preferential loss of sialic acids during ionization and CID experiments. MALDI in negative ion mode was then used to select peaks of derivatized ions of $[M^{**}-H]^-$. The CID spectra of isomeric disialylated SGP showed distinct spectral patterns, which enabled clear discrimination between α2,3- and α2,6-sialylation. Interestingly, the approach is applicable to both the disialylated and monosialylated form. The unique fragmentation behavior is derived from the difference of pyrene-binding positions after ionization between α2,3- and α2,6-sialylated glycopeptides. In conclusion, we have developed a novel method to distinguish isomers of sialylated glycopeptides using an on-plate PDAM derivatization procedure followed by negative-ion MS^2.

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